From Funny Current to HCN Channels: 20 Years of Excitation

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The “funny” (pacemaker) current has unusual characteristics, including activation on hyperpolarization, permeability to K+ and Na+, modulation by internal cAMP, and a tiny, single-channel conductance. In cardiac cells and neurons, pacemaker channels control repetitive activity and excitability. The recent cloning of HCN subunits provides new insight into the molecular basis for the funny channel properties.

The pacemaker function of the specialized myocytes of cardiac conduction tissue resides in the ability of these cells to generate a diastolic (“pacemaker”) depolarization. At the termination of an action potential, the pacemaker depolarization slowly brings the membrane voltage up to threshold for initiation of a new action potential, thus producing repetitive activity (Fig. 1A).

The ionic mechanism underlying the pacemaker depolarization is the activation of the hyperpolarization-activated current ($I_f$). When first described in the rabbit cardiac sinoatrial node (SAN), the natural pacemaker region of the heart, $I_f$ was defined as the “funny” current because of several unusual features (7). One unusual feature is its voltage dependence. $I_f$ is activated by hyperpolarizations (Fig. 1B) with a threshold of approximately −40/−50 mV in the SAN. Figure 1C shows a typical activation curve, which reports the relative fraction of channels open at steady state as a function of membrane voltage. This relation indicates that the current is activated at voltages comprising the range of the diastolic depolarization (compare with Fig. 1A). The fully activated current/voltage relation reverses near −10/−20 mV in physiological solutions as a consequence of the channel mixed permeability to Na+ and K+, a second unusual property of $I_f$ (7). The activation by hyperpolarization and permeability to Na+ and K+ are critical properties with respect to the role of $I_f$ in the generation of diastolic depolarization and hence of spontaneous activity. Thus “f channels” are opened by hyperpolarization in the pacemaker range of voltages and carry the inward current, which generates the diastolic depolarization, eventually leading to the threshold for Ca2+ channel activation and action potential firing.

Autonomic stimulation alters the voltage dependence of $f$ channel activation but not the fully activated current, leading to changes in the slope of the diastolic depolarization and heart rate. Figure 1A shows the effects of low concentrations of autonomic agonists, which clearly affect $I_f$ (Fig. 1B), on spontaneous action potentials. The slope of the diastolic depolarization is altered with little effect on the shape or duration of the action potential, resulting in changes in heart rate. β-Adrenergic agonists increase $I_f$ at diastolic potentials by shifting the activation curve to more positive voltages (Fig. 1C). This shift provides more inward current at diastolic potentials, increasing the slope of the diastolic depolarization and accelerating heart rate. Muscarinic agonists have opposite effects on $I_f$ and shift the activation curve to more negative voltages (7). Thus less inward current is available at diastolic potentials, causing a decreased slope of this phase and decelerating heart rate. The shift in the activation curve, in response to isoproterenol and acetylcholine, is demonstrated in Fig. 1C. The actions of catecholamines and acetylcholine on $I_f$ thus play a key role in underlying cardiac rate modulation by autonomic stimuli (7).

How do autonomic transmitters modulate $I_f$? β-Adrenergic agonists are known to increase intracellular cAMP levels by activating adenylate cyclase, and the idea that $I_f$ acts as a second messenger in the modulation of the pacemaker current was proposed in early studies, when this current was still wrongly interpreted as a pure K+ current. Following the finding of $I_f$ in the SAN and its reinterpretation in Purkinje fibers, this problem was best addressed by inside-out experiments in which the intracellular sides of f channels were exposed to the perfusing solution. Application of cAMP to the inner membrane side of macropatches excised from SAN myocytes shifted the activation curve of $I_f$ to more positive voltages due to a direct interaction of intracellular cAMP with f channels, according to a phosphorylation-independent mechanism (7). The direct effect of cAMP, like the action of autonomic neurotransmitters, did not modify the $I_f$ fully activated conductance. The cAMP-induced shift ranges from −11 to 14 mV in the SAN, accounting for most of the additive shift of −18 mV produced by maximal stimulation with β-adrenergic and muscarinic agonists (1) (Fig. 1C). A half-maximal shift is obtained with a cAMP concentration of 0.2 μM.

The f channels were the first described example of channels that could be modified simultaneously by voltage, as for voltage-gated channels, and by the binding of cAMP, as for cyclic nucleotide-gated channels. Modulation by cAMP can be interpreted according to a cyclic allosteric channel activation model, whereby cAMP binds preferentially to open channels and locks them in an open state (6). The allosteric hypothesis readily accounts for the cAMP dependence of the shift in channel activation by assuming a nearly sixfold higher affinity of the molecule for open than for closed channel configurations.
Recent experiments have investigated the contribution of intracellular regions to channel properties. The application of pronase to excised patches from SAN myocytes shifts the activation curve of \( I_f \) to more depolarized potentials by \(-57 \) mV without affecting the fully activated relation (3). In addition, the effects of cAMP on the current are completely abolished. These findings suggest that an inhibitory mechanism located at the COOH terminus, where cAMP binding occurs, is involved in channel gating and that this mechanism is removed partly by cAMP binding and completely by pronase-induced cleavage of the COOH terminus. This leads to the hypothesis that the COOH terminus of \( f \) channels contributes to gating by inhibiting channel opening and that the shifting action of cAMP is due to attenuation of the inhibitory process (Fig. 2).

**Hyperpolarization-activated cyclic nucleotide-gated channel subunits**

Despite the importance of the pacemaker channel to functional properties of cardiac myocytes (7), its cloning was only achieved nearly three decades after its original description and was accomplished by chance. A putative member of a new family of channels was indeed cloned from mouse brain by using the yeast two-hybrid approach while looking for proteins interacting with the SH3 binding domain of neural Src (13). These channels are now known as hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Using complementary techniques of BLAST searches of expressed sequence tag database, RT-PCR, and screening of cDNA libraries, four isoforms (HCN1–4) were cloned in mammals (8, 9, 14, 18). The functional expression of these channels resulted in currents with the hallmarks of the cardiac \( I_f \) or of its neuronal equivalent (termed \( I_f \)). Although the properties of different HCN isoforms differ quantitatively (see below), all isoforms yield currents that are activated by hyperpolarization, carry K\(^+\) and Na\(^+\), are blocked by Cs\(^+\) in a voltage-dependent way, and are modulated by a direct action of cAMP on the cytoplasmic side of the channel (2, 9, 10, 14).

HCN channels have a structure and proposed topology typical of voltage-gated K\(^+\) channels (Fig. 3A). The primary structure indicates six transmembrane segments, a positively charged S4 segment, and the GYG pore sequence found in most known K\(^+\)-selective channels. HCN channels also exhibit a high similarity to cyclic nucleotide-gated channels in the cyclic nucleotide-binding domain located in the COOH terminus.

The similarities of HCN channels with voltage-gated K\(^+\) channels raises two questions concerning the nature of structure-function relation in these channels. For example, the HCN S4 segment has two regions (a “double-barreled” S4), each containing five positively charged amino acids. This finding is puzzling since, in contrast to voltage-gated K\(^+\) channels that also carry positively charged S4 segments, HCN channels open on hyperpolarization. Another puzzling feature of HCN channels is their high Na\(^+\) permeability, which appears to contrast with the GYG pore sequence typical of many K\(^+\)-selective filters. The unusual biophysical properties of HCN channels remain to be fully resolved.

**HCN channel kinetics**

The currents produced by expression of HCN1, HCN2, and HCN4 isoforms are activated by hyperpolarization but have different relative rates of activation and deactivation. HCN1 has the fastest kinetics, followed by HCN2 and HCN4 (2). Currents recorded in HEK-293 cells expressing mouse HCN1, human HCN2, and rabbit HCN4 and the corresponding mean activation curves and activation time constant curves are shown in Fig. 3B. The half-maximal voltage (\( V_{1/2} \)) of the mean activation curves in Fig. 3B is about \(-73 \) mV for HCN1, \(-92 \) mV for HCN2, and \(-81 \) mV for HCN4. Values of \( V_{1/2} \) reported in the literature are variable, but in general they indicate a more negative position for HCN2 than for HCN4 and HCN1 (5, 8, 9, 10, 16).

CAMP modulates HCN channels by the same mechanism operating with \( f \) channels, i.e., by a shift of the voltage dependence of activation resulting from a direct interaction with the channel. The presence of a cyclic nucleotide-binding domain
in the sequence of HCN channels confirms at a molecular level the “direct” versus the “phosphorylation-dependent” activation mechanism proposed for native f channels (7).

Concerning the molecular basis for the cAMP-dependent gating, the concept of a COOH terminus-mediated inhibitory mechanism relieved partially by cAMP, as represented in the model scheme of Fig. 2 for native I_f, has been confirmed by site-directed mutagenesis of the COOH linker of HCN1 and HCN2 isoforms (19).

Although the basic channel activation mechanism is likely to be the same, the efficacy of cAMP action has been found to differ between isoforms, with HCN1 being far less responsive than HCN2 and HCN4. Maximal shifts of the activation curve obtained in either whole cell or inside-out recordings vary in the following ranges: 2–6.7 mV (HCN1); 12–15 mV (HCN2); and 15.2–23 mV (HCN4) (8, 9, 10, 11, 13, 14).

The half-activation concentration of cAMP on mHCN2 was 0.5 μM (9), a value close to that of 0.2 μM found in the rabbit SAN (7). These results support the idea that the effect of cAMP on HCN2 and HCN4, more than that on HCN1, is similar to the effect on I_f in the SAN. Interestingly, however, HCN1 is expressed in cardiac sinoatrial but not atrial or atrioventricular cells (11). Finally, mHCN2 is ~10 times less sensitive to cGMP compared with cAMP (9). This is similar to the relative sensitivity of I_f in the SAN to cGMP and cAMP (7).

As in voltage-gated K⁺ channels, the S4 domain of HCN channels may act as a voltage sensor and may be linked to the “intrinsic” gating mechanism of I_f as proposed in experiments using pronase (3). The involvement of S4 domains in sensing voltage has been shown by experiments in which nine basic residues and a single serine in the S4 segment were mutated individually, or in combination, to glutamine (4). Individual mutations of Lys²⁹¹, Arg²⁹⁴, Arg²⁹⁷, and Arg³⁰⁰, located in the outermost portion of the S4 domain, to glutamine produced a shift in the voltage dependence of opening to more hyperpolarized potentials. Combining all mutations produced a cumulative effect on the position of the activation curve. Similar results were obtained in other studies (17) in which hyperpolarizing shifts in the activation curve were produced by neutralizing mutations of Lys²⁹¹ and Arg³⁰⁰. They further showed that a substitution of Lys²⁹¹ with a negatively charged amino acid more than doubled the hyperpolarizing shift. Strangely, there were no effects of these mutations on the slope of the activation curve, which, according to Boltzmann distribution analysis, should represent the net gating charge moved across an electric field during channel opening.

Recently, the allosteric hypothesis, first introduced to explain the cAMP dependence of gating in native channels, has been extended to include voltage-dependent gating of HCN channels (Fig. 4). This model suggests that voltage and cAMP use a common mechanism to increase the channel open probability (2). According to the allosteric model, channel opening is the combination of two processes: 1) displacement of voltage sensors (one for each of the four subunits of a tetrameric channel) from “reluctant” to “willing” states and 2) allosteric closed-to-open transitions involving “concerted” rearrangements of all four subunits. The probability of channel opening increases every time one voltage sensor switches to

![FIGURE 2. cAMP-dependent gating model for f channels. Top: a voltage-dependent gating mechanism controls changes from “reluctant” to “willing” channel states. Full opening requires a second configuration change [from closed (C) to open (O)], which depends on removal of an inhibitory mechanism (depicted conventionally as a blocking “ball”) involving the COOH terminus (top right). Disinhibition and opening are favored by cAMP binding to the cyclic nucleotide binding domain (on the COOH terminus) according to an allosteric cAMP-dependent gating model; this only requires the assumption that cAMP binding is more likely to occur with open than with closed channels to explain cAMP-dependent shifts of the open probability curve (6). CX, closed channels with bound cAMP; OX, open channels with bound cAMP. Bottom: when the COOH terminus is cleaved by pronase, the cAMP-dependent inhibitory mechanism is removed and channel gating only reflects state changes dependent on reluctant/willing transitions. Modified with permission from Ref. 3.](image)
the “willing” state. The presence of two steps in channel gating, a “priming” process involving the movement of voltage sensors and a proper closed-to-open transition, accounts for several kinetic features, including the “delay” in current activation and its removal by preconditioning short, high hyperpolarizing steps and the delay in current deactivation during depolarization, which cannot be explained by simple Hodgkin-Huxley mechanisms. Interestingly, the allosteric hypothesis also accounts for the lack of change in the slope of the activation curve in the S4 mutation experiments discussed above, since change of gating charge of sensor displacements does not directly affect the closed-to-open transitions. The model also provides a physical explanation for the different kinetic features of the different isoforms. For example, the faster kinetics of HCN1 activation may be explained by a faster activation of the voltage sensor as well as a looser interaction between the subunits in the closed-to-open transition.

**Ionic properties of HCN channels**

As in native channels, the fully activated current/voltage relation of HCN channels is linear and reverses at potentials compatible with permeability to both Na⁺ and K⁺, with a preference for K⁺. Values of \( \frac{P_{\text{Na}}}{P_{\text{K}}} \) ratio ranging from 0.25 to 0.41 have been reported for the cloned channels (8, 9, 10).

HCN channels are blocked in a voltage-dependent way by external Cs⁺. The voltage dependence can be interpreted by assuming that Cs⁺ blocks after crossing ~66% of the electrical field to reach its binding site. This fraction is similar to that of 71% found for Cs⁺ block of \( I_f \) measured in Purkinje fibers (10). On the other hand, the concentration of Cs⁺ required to block 50% of current was 15 mM for HCN2 channels, compared with 2.2 mM in Purkinje fibers, implying that channels in this tissue are unlikely to be composed of HCN2 subunits only. However, in the SAN, even 5 mM Cs⁺ leaves a large fraction of current unblocked at diastolic potentials, suggesting some variation between tissues, perhaps related to isoform expression (see Refs. 7 and 10 for further discussion and references).

**Heteromultimeric assembly of HCN channels**

HCN isoforms have been shown to coassemble and form heteromers. Expression of concatamers of mHCN1 and mHCN2 has shown that currents with kinetics intermediate to those obtained by expression of each isoform alone are produced (5, 16). These results indicate that heteromultimeric channels can be formed by cells that express both the HCN1 and HCN2 genes, in addition to the formation of homomeric channels. Interestingly, each subunit exerted dominant effects on certain characteristics of HCN1-HCN2 heteromultimeric channels. Specifically, the midpoint of activation was close to that of HCN2 channels, whereas the speed of activation reflected more closely the properties of the HCN1 subunit. The shifts in HCN1-HCN2 channels produced in response to cAMP seem most similar to the shifts observed with HCN2 alone, i.e., the effects of cAMP reflected primarily those of HCN2. Clearly, a comprehensive examination of all channel properties must be carried out with heteromultimeric assem-

blies of HCN channels of varying isoform composition, since they cannot be simply extrapolated from those of the individual components.

More recent data suggest the possibility that native pacemaker channels include β-subunits as well as HCN subunits (20). Coinjection of mink-related peptide 1 (MIRP1) with HCN1 or HCN2 isoforms increased the magnitude of expressed currents, suggesting that MIRP1 provides targeting and/or stabilization of the channel complex. Since coexpression with MIRP1 modifies activation/deactivation kinetics of HCN isoforms, the presence of a β-subunit contributes to kinetic variability of pacemaker channels.

**The relationship of isoform distribution with the presence of \( I_f \)-like currents**

HCN channels are highly expressed in the heart. HCN4 was originally cloned from the SAN (8), whereas HCN2, HCN3, and HCN4 have been found in the heart using Northern blot analysis and screening of cardiac cDNA libraries (9, 14). HCN2 mRNA has been found in the mouse ventricle (9), whereas the murine SAN has been shown to contain HCN1, HCN2, and especially HCN4 (12). Studies using RNase pro-

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tection assays have found a dominant amount of HCN4 mRNA and lesser amounts of HCN1 mRNA in the SAN, equal amounts of HCN1 and HCN4 with a smaller amount of HCN2 mRNA in Purkinje fibers, and primarily HCN2 mRNA in the ventricles (15).

Recent studies describing the cloning and expression of rabbit HCN1 have found that this protein is present in individual myocytes of the rabbit SAN (11). This represents the first direct confirmation of HCN channel protein in cardiac myocytes. These findings indicate that different parts of the heart may express multiple HCN isoforms and produce currents that differ from those produced by heterologous expression of individual isoforms. The presence of HCN1 protein in SAN myocytes may appear perplexing, since If in the SAN resembles currents produced by expression of HCN2 and HCN4 currents more closely than those produced by HCN1 (8, 10).

It is, however, interesting to note that heteromultimers formed by assembly of HCN1 with HCN2 channels have faster kinetics than homomeric HCN2 channels (5, 16). If a similar situation applies to heteromultimerization of HCN1 and HCN4, this might for example serve to provide “central” node cells with a slightly faster pacemaker current. Hence these cells will have a beating rate slightly faster than that of more peripheral cells, an essential feature of the true “pacemaker” cardiac region. Ruling in favor of the heteromeric hypothesis is evidence that the activation kinetics of native If current in the SAN are intermediate between those of HCN1 and HCN4, the two isoforms with a significant degree of expression in this tissue (11).

Clearly, the relative amounts of protein for each isoform making up individual channels, the presence of β-subunits, and the total number of homomeric and heteromeric channels expressing each isoform must be determined for each tissue. The consequences of heteromultimerization on all properties of channels made up of different isoforms present in varying proportions must also be determined. This may include the potential for unique sets of posttranslational modifications of channel structure and/or for unique channel trafficking, in addition to distinct biophysical properties.

Conclusions and perspectives

The knowledge of the primary structure of HCN channels now allows for the study of the molecular determinants of the “funny” features of native pacemaker channels as well as their expression in native tissue. The properties investigated include activation by hyperpolarization, selectivity for both Na+ and K+, and modulation by cAMP. The structural basis of these characteristics is now being examined using site-directed mutagenesis in combination with patch clamp studies. Although S4 segments are clearly involved, more studies are needed to understand the mechanisms of gating in response to voltage. These mechanisms, and their differences between subunits, may be more easily understood in the context of an allosteric model describing both cAMP and voltage-dependent gating.

The evidence that different isoforms, along with β-subunits, can assemble to form heteromultimeric channels with modified properties (5, 16, 20) may provide a key to understanding why the properties of native channels often differ from channels consisting of individual isoforms have quantitatively different features that may be further influenced by β-subunits. Coassembly of different α- and β-subunits may represent a way to modulate such properties as the rate of current activation on hyperpolarization and cAMP sensitivity in the various tissues where HCN channels are coexpressed.

Structure-function information will also be important for understanding how naturally occurring mutations in the primary sequence of these channels may affect the function of the channel. Since If channels serve several different functionally relevant roles in many excitable cells, this approach will help provide the molecular basis for physiological and potential pathological roles.
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